

The University of Chicago

THE BIURET REACTION

III. THE BIURET REACTION OF AMINO ACID AMIDES

A PART OF A DISSERTATION SUBMITTED TO
THE FACULTY OF THE DIVISION OF THE
PHYSICAL SCIENCES IN CANDIDACY FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF CHEMISTRY

By

PETER SUHSUN YANG

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An investigation of the chemistry of the biuret reaction was begun in recent years by one of the authors (1, 2) to ascertain the nature and constitution of the colored products of the reaction, and the atoms in molecules showing the biuret reaction which take part in, and are essential for, the occurrence of the reaction. It is hoped that the data obtained from this study will furnish ultimately some clues regarding the molecular structure of the proteins, substances which so characteristically show the biuret reaction. The work just mentioned continues that of Schiff (3), who investigated qualitatively the behavior of a large number of compounds with the biuret reagents, alkali and cupric ion, and who was the first to isolate a product of the reaction, potassium copper biuret, $K_2^+(Cu(biuret)_2) \cdot 4H_2O$.

The investigations by Rising and her collaborators have demonstrated certain interesting facts regarding the formulas of the biuret reaction salts of di-acid amides and acid imides, and have led to the development of some useful theories concerned with the atoms which take part in the reaction, the structure of the salts formed, and the rôles of the biuret reagents. Some of these facts and theories have been discussed in previous papers (1, 2). The results of the study of the biuret reaction of amino acid amides, now to be described, offer further support for these earlier

* The work here described forms part of the dissertation of Peter S. Yang, presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy at the University of Chicago.

The contents of this paper were reported at the national meeting of the American Chemical Society at Cincinnati, September, 1930.

theories, and indicate that the application of these theories is by no means limited to the biuret reaction of di-acid amides and acid imides. The experimental results augment the facts already ascertained regarding the chemical nature of the biuret reaction.

The investigation of the biuret reaction of α -amino acid amides, compounds found by Schiff to show the reaction, was undertaken as a logical step leading to the study of the reaction of the proteins with the biuret reagents. The amides of *dl*-leucine, *l*-aspartic acid, *d*-alanine, and glycine were prepared (4), each amide was treated with the biuret reagents, and the colored "biuret salts" of the amides were isolated and analyzed. This study has brought to light certain likenesses, as well as some striking variations, in the behavior of amino acid amides with alkali and copper salts, as compared with the action of di-acid amides and acid imides under the same conditions. The biuret reaction of amino acid amides is less complex than that of the other types of compounds mentioned in that the reaction of the former occurs in the absence of alkali, producing internal salts having the empirical formula $(\text{Cu}(\text{amino acid amide})_2) \cdot x\text{H}_2\text{O}$. Products of this formula were formed when alkali was used and when it was not used, and no product of the biuret reaction of any amino acid amide studied contained alkali. In contrast to biuret salts of this type are those of di-acid amides, of empirical formula¹ $\text{Me}_2^+(\text{Cu}(\text{di-acid amide})_2) \cdot x\text{H}_2\text{O}$,² and of acid imides, of formula³ $\text{Me}_2^+(\text{Cu}(\text{acid imide})_4) \cdot x\text{H}_2\text{O}$ (5), in which Me^+ represents Na^+ or K^+ .

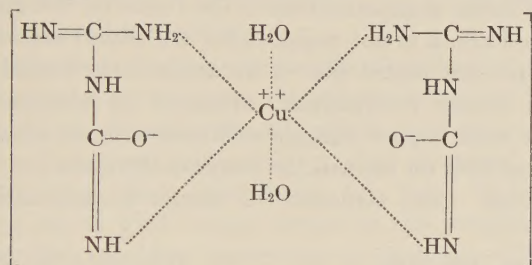
Many compounds which show the biuret reaction do so only in the presence of an excess of a strong base. The positive reactions of amino acid amides in the absence of alkali are not, however, the first instances to be noted of the occurrence of the biuret reaction without alkali. Monoiminobiuret, $\text{NH}_2 \cdot \text{CO} \cdot \text{NH} \cdot \text{C}-$

¹ Because of the instability of "biuret salts," their molecular weights have been difficult to determine. This has, however, been accomplished for potassium copper succinimide, and the data obtained prove that the molecular, as well as the empirical, formula of this salt is $\text{K}_2^+(\text{Cu}(\text{succinimide})_4) \cdot 6\text{H}_2\text{O}$. This work, by Mary M. Rising, L. B. Jefferies, and C. C. Li, will presently be published.

² Proved by Schiff to be correct for potassium copper biuret, and confirmed by Rising, Hicks, and Moerke for other di-acid amides.

³ This formula has been confirmed by Rising and Johnson for the acid imide diethylbarbituric acid.

(:NH)NH₂, and diiminobiuret, NH₂·C(:NH)NH·C(:NH)NH₂, react with copper salts in the absence of alkali to form deep red products of formula (Cu(iminobiuret)₂)·xH₂O, and are considered to show the biuret reaction (6). A constitution for the biuret salt of iminobiuret was first proposed by Rising and Johnson (1), which it is now desired to amend. The improved structure suggested follows.



Copper monoiminobiuret

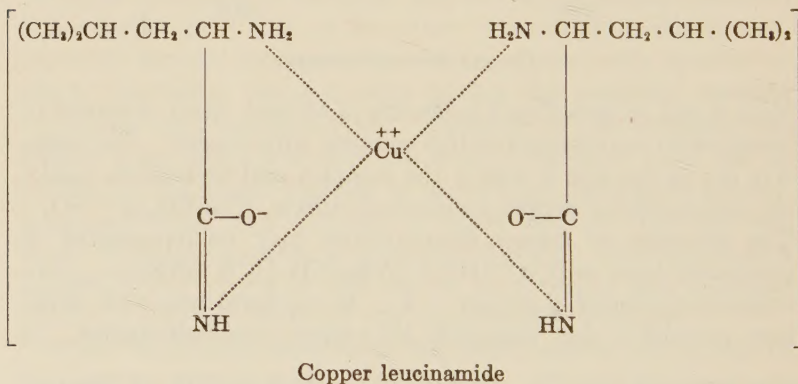
Here it will be seen that 2 molecules of an enol (acid) tautomer of biuret enter an ammonium-like complex with copper. The negative ion of the salt is within the complex and un-ionized, unlike the acid radical of copper ammonium sulfate, (Cu(NH₃)₄)⁺⁺ SO₄⁼. The structure of copper diiminobiuret may be represented in condensed form as (Cu⁺⁺(HN:C(NH₂)NH·C(:N⁻)NH₂)₂). (Coordination number of copper = 4.) In a general way this structure resembles that suggested for copper monoiminobiuret. It

will be noted that the amidine group, H₂N·C:NH, of diiminobiuret is considered to function as does the amide group, H₂N·C:O, of monoiminobiuret, but the latter must enolize before salt formation can occur. The amide group is probably more strongly acid than is the amidine group and would therefore react if present, but when no amide group occurs in a molecule showing the biuret reaction, as in diiminobiuret, the amidine group must perforce function.⁴ The central nitrogen atom of the biuret molecule is

⁴ Amidines are known to form salts with bases in which the hydrogen atom of a —NH group is replaced by a metal ion, *e.g.* silver diphenylbenzamidine, C₆H₅·C(=NC₆H₅)N⁻ (C₆H₅)Ag⁺.

not believed to be involved in the biuret reaction; this conclusion has been drawn from the fact that malonamide, $\text{NH}_2 \cdot \text{CO} \cdot \text{CH}_2 \cdot \text{CO} \cdot \text{NH}_2$, containing a carbon atom instead of the central nitrogen atom of biuret, reacts with the biuret reagents exactly as does biuret itself.

There is a distinct parallel in the behavior of the iminobiurets and α -amino acid amides with the biuret reagents, since the biuret salts of the latter compounds have the formula, $(\text{Cu}(\text{amino acid amide})_2)$, and alkali is not required for the biuret reaction of the amides. The first biuret salt of an amino acid amide prepared by us was copper leucinamide, obtained by treatment of *dl*-leucinamide with copper acetate with and without alkali. From the analytical data for the salt the empirical formula $\text{CuC}_{12}\text{H}_{26}\text{N}_4\text{O}_2$ was calculated. The structure of copper leucinamide may be



2 molecules of enol leucinamide enter a complex with copper. The negative radical of the salt is composed of the 2 enol ions, and is within the complex. It was anticipated that salts of this type should not conduct a current, since the ions are not free to move to the electrodes. This prediction was verified by experiment. Electrolysis of a neutral solution of copper leucinamide led to no conductance of the current and no migration of a colored ion to either electrode.

Electrolysis of a solution of copper leucinamide containing a small amount of alkali resulted in the migration of a colored ion to the anode. The effect of alkali upon copper leucinamide may

be to form a soluble compound, potassium copper leucinamide hydroxide, of formula $K_2^+(Cu(OH)_2((CH_3)_2CH \cdot CH_2 \cdot CH(NH_2) \cdot CO-(\cdot NH))_2)$, somewhat as copper ammonium sulfate reacts with alkali to form copper ammonium hydroxide. In the former case the base must be un-ionized since the colored ion is negative, moving to the anode. That this substance was not formed instead of copper leucine when leucine was treated with alkali and copper acetate is in all probability due to the relative insolubility of copper leucinamide.

d-Alaninamide and glycineamide, like leucinamide, react with cupric ion to form colored salts of empirical formulas $CuC_6H_{14}N_4O_2$ and $CuC_4H_{12}N_4O_3$ respectively, and alkali is not required for these reactions. Structures analogous to that proposed for copper leucinamide have been formulated for these biuret salts. *l*-Asparagine reacts with copper acetate in the presence or absence of alkali to form a product containing no alkali ion. The empirical formula of copper asparagine is $CuC_5H_{14}N_4O_6$. This substance does not conduct a current in solution. The salt may be a true biuret reaction product of structure $(Cu^{++}(CO_2-CH(N^+H_3)-CH_2 \cdot CO-(\cdot NH))_2)$. (Coordination number of copper = 2.) Or the compound may be a complex copper salt of the carboxylic acid asparagine, having the structure $(Cu^{++}(CO_2-CH(NH_2)-CH_2 \cdot CO \cdot NH_2)_2)$. (Coordination number of copper = 4.) In neither case are the ions free to conduct the current.

It is possible that the molecule of asparagine, which contains a free hydroxyl group, is neutralized by alkali when the amide is treated with cupric ion in the presence of a strong base and that a soluble salt, *e.g.* potassium copper asparagine, is formed. The compound copper asparagine may then be produced at the expense of the alkali copper salt, due to its greater insolubility.

The empirical formulas established for the biuret salts of the various types of compounds showing the biuret reaction, and studied so far, are set down here for ready inspection.

Empirical Formulas

Biuret salts of di-acid amides,	$Me_2^+ (Cu \text{ (di-acid amide)}_2)^- \cdot xH_2O$
“ “ “ acid imides,	$Me_2^+ (Cu \text{ (acid imide)}_4)^- \cdot xH_2O$
“ salt “ tetrapeptide (7),	$Me_2^+ (Cu \text{ (peptide)})^- \cdot xH_2O$
“ salts “ amino acid amides,	$(Cu \text{ (amino acid amide)}_2) \cdot xH_2O$
“ “ “ iminobiurets,	$(Cu \text{ (iminobiuret)}_2) \cdot xH_2O$

It is undoubtedly significant that, in the cases so far studied, irrespective of the presence or absence of alkali in the biuret salt, the aggregate of molecules in the complex is just sufficient to make available 4 basic nitrogen atoms for taking part in the biuret reaction. These 4 nitrogen atoms may all be present in 1 molecule, as in the tetrapeptide to which reference has just been made, or in 2, or 4, molecules of the reacting substance, as indicated in the formulas.

The minimum number of acid hydrogen atoms involved in the biuret reaction seems to be 2, as in the case of the iminobiurets and amino acid amides. These compounds are strongly basic and no neutralization of acid groups by alkali is required to favor complex ion formation by the enhancing of the basic character of the nitrogen atoms. Biuret salts of such compounds therefore contain no alkali. When more weakly basic molecules showing the biuret reaction contain acid groups which require neutralization in order to increase the basic strength of the compound, as do the di-acid amides, acid imides, and peptides, 4 acid hydrogen atoms take part in the reaction, 2 of them reacting with alkali, and the biuret salts contain alkali. For the biuret reaction of such compounds salt formation is believed to be a fundamental preliminary to the occurrence of the reaction. The number of hydrogen atoms involved may very well depend upon the "acid-base balance" within the active molecules.

The development of the theory of the biuret reaction, based upon the results of further experimental work, will be continued.

EXPERIMENTAL

Copper dl-Leucinamide, $CuC_{12}H_{26}N_4O_2$ —Copper *dl*-leucinamide was prepared as follows: (a) Without alkali. Solid *dl*-leucinamide (1.5 gm.), prepared by the method of Yang (4), was treated with a solution of copper acetate (2.2 gm. in 30 cc. of water). A pasty red substance formed and settled out completely upon the addition of a little water to the reaction solution. The compound was brought upon a filter, washed with water, alcohol, and ether successively, and dried over sulfuric acid. The yield was 1.1 gm. The analytical data follow (the copper content of this salt and of the others to be described later was determined by electrolysis.

Sodium was determined as sodium sulfate in solutions of the salts from which copper had been removed).

0.1159, 0.1105 gm. substance: 0.0226, 0.0218 gm. Cu

$\text{CuC}_{12}\text{H}_{26}\text{N}_4\text{O}_2$. Calculated. Cu 19.72

Found. " 19.49, 19.72

0.0541, 0.0520 gm. substance: 0.0891, 0.0859 gm. CO_2 , 0.0409, 0.0388 gm. H_2O

$\text{CuC}_{12}\text{H}_{26}\text{N}_4\text{O}_2$. Calculated. C 44.75, H 8.13

Found. " 44.91, 45.05, H 8.47, 8.34

0.1960, 0.1895 gm. substance: HCl (factor 0.0978) 24.73, 24.50 cc.

$\text{CuC}_{12}\text{H}_{26}\text{N}_4\text{O}_2$. Calculated. N 17.40

Found. " 17.27, 17.70

(b) With alkali. A solution of *dl*-leucinamide (0.5 gm. in 20 cc. of water) was treated with copper acetate (0.77 gm. in 10 cc. of water), the reaction mixture being stirred. The solution became reddish purple in color. A 40 per cent aqueous solution of potassium hydroxide (1 cc.) was then added drop by drop, whereupon a red precipitate formed. This was brought upon a filter, washed with very dilute ammonium hydroxide to remove copper hydroxide, then with water, alcohol, and ether. The salt was dried over sulfuric acid. The yield was 0.5 gm. The compound was analyzed and contained no potassium. The analytical data follow.

0.0636, 0.0507 gm. substance: 0.0125, 0.0100 gm. Cu

$\text{CuC}_{12}\text{H}_{26}\text{N}_4\text{O}_2$. Calculated. Cu 19.72

Found. " 19.65, 19.72

0.0545, 0.0788 gm. substance: 0.0894, 0.1290 gm. CO_2 , 0.0397, 0.0574 gm. H_2O

$\text{CuC}_{12}\text{H}_{26}\text{N}_4\text{O}_2$. Calculated. C 44.75, H 8.13

Found. " 44.73, 44.64, H 8.14, 8.15

0.0636, 0.0507 gm. substance: HCl (factor 0.0978) 8.19, 6.63 cc.

$\text{CuC}_{12}\text{H}_{26}\text{N}_4\text{O}_2$. Calculated. N 17.40

Found. " 17.60, 17.90

The analytical data indicate that the biuret salts of leucinamide obtained in the presence or absence of alkali are identical. This identity is shown further by the behavior of the two products. Each is a reddish amorphous substance which melts with decomposition at 248–250° (uncorrected) (8).⁵ They are rather in-

⁵ Bergell and Brugsch isolated a red copper derivative of leucinamide of melting point 222–223° (corrected). Analytical data: Cu 19.70, C 29.69, N 17.25, H 8.14.

soluble in water and in alcohol. The analytical data agree well with the empirical formula $\text{CuC}_{12}\text{H}_{26}\text{N}_4\text{O}_2$. The structure proposed for copper leucinamide is to be found in the introduction to this paper. This structure is supported by the behavior of the salt upon electrolysis. Its aqueous solution (50 mg. of the salt in 30 cc. of water) does not conduct a current.

Copper l-Asparagine, $\text{CuC}_8\text{H}_{14}\text{N}_4\text{O}_6$ —Copper *l*-asparagine was prepared by treatment of *l*-asparagine with copper acetate, and the product of the reaction was the same whether obtained in the presence or absence of alkali. When, for example, 2 gm. of powdered asparagine were suspended in 30 cc. of water and treated with concentrated aqueous potassium hydroxide until the amide dissolved and the solution became alkaline, the addition of saturated aqueous copper acetate dropwise to the alkaline solution resulted in the appearance of a purplish blue color. With continued addition of the copper acetate solution, precipitation of lavender copper asparagine occurred. Copper acetate solution was added until the precipitation of the biuret salt was complete; at this time the color of the solution had changed to blue. Some 20 minutes were allowed for complete precipitation; the salt was brought upon a filter, washed with a little 20 per cent potassium bicarbonate solution to remove copper hydroxide, then thoroughly with water, and was finally dried over sulfuric acid and analyzed. It contained no potassium.

0.1525, 0.1357 gm. substance: 0.0296, 0.0260 gm. Cu

$\text{CuC}_8\text{H}_{14}\text{N}_4\text{O}_6$. Calculated. Cu 19.52

Found. " 19.40, 19.16

0.1484, 0.2178 gm. substance: 0.1593, 0.2335 gm. CO_2 , 0.0594, 0.0858 gm. H_2O

$\text{CuC}_8\text{H}_{14}\text{N}_4\text{O}_6$. Calculated. C 29.48, H 4.30

Found. " 29.28, 29.24, H 4.47, 4.40

0.2573, 0.1923 gm. substance: HCl (factor 0.0987) 32.14, 24.41 cc.

$\text{CuC}_8\text{H}_{14}\text{N}_4\text{O}_6$. Calculated. N 17.20

Found. " 17.65, 17.40

Copper *l*-asparagine is a lavender amorphous substance which chars at 302–304° (uncorrected). The salt is rather insoluble in water and alcohol. The analytical data agree well with the empirical formula $\text{CuC}_8\text{H}_{14}\text{N}_4\text{O}_6$. As stated in the introduction

the compound may be a true biuret reaction product, or it may be a complex copper salt of the carboxylic acid asparagine.

Copper Glycinamide, $\text{CuC}_4\text{H}_{12}\text{N}_4\text{O}_3$ —This salt was obtained (1) by treatment of glycinamide with copper acetate in the presence of alkali, and (2) by treatment of the amide with copper hydroxide in the absence of alkali. The products obtained by the two methods are identical and contain no alkali ion. When method (1) was used, glycinamide (1 gm.) was dissolved in 10 cc. of water and to this solution were added, with mechanical stirring, 3.9 cc. of 20 per cent aqueous sodium hydroxide. The reaction mixture was cooled meanwhile in an ice and salt bath. Then 10 cc. of an aqueous solution containing 1.8 gm. of anhydrous copper sulfate were added to the mixture drop by drop. The purple reaction solution was stirred for 2 hours, filtered, cooled in ice, and treated with 100 cc. of dry alcohol to precipitate potassium sulfate. When this salt was separated by filtration of the reaction mixture, 200 cc. of dry ether were added to the filtrate to precipitate copper glycinamide. The salt was brought upon a filter, washed with alcohol and ether, dried over phosphorus pentoxide, and analyzed. The yield was 0.8 gm.

0.0767, 0.0895 gm. substance: 0.0213, 0.0249 gm. Cu

$\text{CuC}_4\text{H}_{12}\text{N}_4\text{O}_3$. Calculated. Cu 27.91

Found. " 27.77, 27.82

0.1089, 0.0828 gm. substance: 0.0849, 0.0654 gm. CO_2 , 0.0519, 0.0429 gm. H_2O

$\text{CuC}_4\text{H}_{12}\text{N}_4\text{O}_3$. Calculated. C 21.08, H 5.31

Found. " 21.26, 21.54, H 5.33, 5.43

0.0790, 0.0902 gm. substance: HCl (factor 0.1128) 12.18, 13.92 cc.

$\text{CuC}_4\text{H}_{12}\text{N}_4\text{O}_3$. Calculated. N 24.61

Found. " 24.35, 24.37

Analysis of the copper glycine obtained by treatment of glycinamide with copper hydroxide without alkali gave data which agree with those just stated. All of the analytical results indicate an empirical formula $\text{CuC}_4\text{H}_{12}\text{N}_4\text{O}_3$, or $(\text{Cu}(\text{glycinamide})_2) \cdot \text{H}_2\text{O}$ for the salt. The compound obtained with and without alkali is a bluish pink amorphous substance having a decomposition point of $205\text{--}207^\circ$ (uncorrected). It is readily soluble in water, and less so in alcohol. Aqueous solutions of the salt do not conduct a current.

Copper d-Alaninamide, $\text{CuC}_6\text{H}_{14}\text{N}_4\text{O}_2$ —This salt was obtained by treatment of *d*-alaninamide with (1) copper acetate in the presence of alkali and (2) copper hydroxide in the absence of alkali. Following the latter plan, a suspension of 0.55 gm. of freshly prepared copper hydroxide in 10 cc. of water was added slowly with mechanical stirring to 1 gm. of alaninamide dissolved in 10 cc. of water. The reaction mixture became deep purple in color. It was stirred for 2 hours, filtered, and treated first with 200 cc. of absolute alcohol, then with 800 cc. of dry ether, whereupon precipitation of orange-red copper alaninamide occurred. The salt was brought upon a filter, washed with dry alcohol and dry ether, dried over sulfuric acid, and analyzed. The yield was 0.50 gm.

0.0572, 0.0750 gm. substance: 0.0152, 0.0201 gm. Cu

$\text{CuC}_6\text{H}_{14}\text{N}_4\text{O}_2$. Calculated. Cu 26.74

Found. " 26.57, 26.80

0.0824, 0.0638 gm. substance: 0.0916, 0.0709 gm. CO_2 , 0.0454, 0.0337 gm. H_2O

$\text{CuC}_6\text{H}_{14}\text{N}_4\text{O}_2$. Calculated. C 30.29, H 5.93

Found. " 30.31, 30.30, H 6.13, 5.91

0.0608, 0.0587 gm. substance: HCl (factor 0.1128) 8.90, 8.70 cc.

$\text{CuC}_6\text{H}_{14}\text{N}_4\text{O}_2$. Calculated. N 23.57

Found. " 23.11, 23.40

The products obtained by methods (1) and (2) were identical, as shown by analytical data, color, and decomposition point (227–231° uncorrected). Results of analysis indicate that the empirical formula of the salt is $\text{CuC}_6\text{H}_{14}\text{N}_4\text{O}_2$. The compound is very soluble in water, and only slightly so in alcohol. Its aqueous solution does not conduct a current.

SUMMARY

1. The "biuret salts" of glycineamide, *d*-alaninamide, *dl*-leucineamide, and asparagine have been isolated and studied.

2. The empirical formula of these biuret salts is $(\text{Cu}(\text{amino acid amide})_2)$.

3. The amino acid amides named show the biuret reaction in the absence of alkali.

4. Results of the work described support the theory that 4 basic nitrogen atoms are involved in a biuret reaction.

5. 2 acid hydrogen atoms take part in the biuret reaction of amino acid amides.

6. The number of hydrogen atoms involved in biuret reactions appears to be 2 or 4, depending upon the acid-base balance of the molecule showing the reaction.

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